

***In vivo* newly translated polypeptides are sequestered in a protected folding environment**

Vanitha Thulasiraman, Chin-Fen Yang and Judith Frydman¹

Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020, USA

¹Corresponding author
e-mail: jfrydman@leland.stanford.edu

Molecular chaperones play a fundamental role in cellular protein folding. Using intact mammalian cells we examined the contribution of cytosolic chaperones to *de novo* folding. A large fraction of newly translated polypeptides associate transiently with Hsc70 and the chaperonin TRiC/CCT during their biogenesis. The substrate repertoire observed for Hsc70 and TRiC is not identical: Hsc70 interacts with a wide spectrum of polypeptides larger than 20 kDa, while TRiC associates with a diverse set of proteins between 30 and 60 kDa. Overexpression of a bacterial chaperonin 'trap' that irreversibly captures unfolded polypeptides did not interrupt the productive folding pathway. The trap was unable to bind newly translated polypeptides, indicating that folding in mammalian cells occurs without the release of non-native folding intermediates into the bulk cytosol. We conclude that *de novo* protein folding occurs in a protected environment created by a highly processive chaperone machinery and is directly coupled to translation.

Keywords: actin/chaperones/chaperonin/protein folding/translation

Introduction

The mechanism by which proteins fold *in vivo* is a central issue in modern biology. The transformation of the one-dimensional genetic information into three-dimensional protein structures depends on the accuracy and efficiency of the process of protein folding. While *in vitro* folding experiments have shown that the process can occur spontaneously, it is now generally accepted that several protein families, generically termed molecular chaperones, are required for the correct folding and assembly of an undetermined number of proteins in the cell (reviewed in Gething and Sambrook, 1992; Hartl, 1996; Bukau and Horwich, 1998).

Two major chaperone systems have been implicated in cytosolic protein folding (Hartl, 1996; Bukau and Horwich, 1998): the 70 kDa heat shock protein cognate (here called Hsc70) and the cytosolic chaperonin TCP1-ring complex (TRiC, also called CCT). The Hsc70 class of molecular chaperones are weak ATPases that bind to peptide segments with a net hydrophobic character (Flynn *et al.*, 1991; Blond-Elguindi *et al.*, 1993; Rudiger *et al.*, 1997).

This results in the stabilization of the polypeptide in an extended conformation and may prevent the premature folding and aggregation of polypeptides during membrane translocation and translation. Binding and release of unfolded polypeptides are nucleotide-dependent events that may result in folding, rebinding to Hsc70 or transfer to other chaperone systems, such as the chaperonins, for final folding (see below). A number of findings are consistent with a requirement of Hsc70 for protein folding in the cytosol: Hsc70 is found in association with ribosome-bound polypeptide chains (Nelson *et al.*, 1993; Frydman *et al.*, 1994; Hansen *et al.*, 1994; Frydman and Hartl, 1996; Pfund *et al.*, 1998) and participates in the folding of some model proteins synthesized in reticulocyte lysate or yeast (Crombie *et al.*, 1994; Frydman *et al.*, 1994; Hansen *et al.*, 1994; Frydman and Hartl, 1996). The chaperonins are oligomeric ring-complexes, composed of ~60 kDa subunits, which mediate the folding of polypeptide chains in an ATP-dependent reaction. In bacteria, Class I chaperonins such as GroEL bind to and fold substrate polypeptides within their central cavity; this process requires the action of the ring-shaped oligomeric cofactor GroES (Hartl, 1996; Bukau and Horwich, 1998). Unlike Hsc70, GroEL does not bind to linear sequence determinants but recognizes more structured folding intermediates, characterized by the solvent-exposure of hydrophobic surfaces (Hayer-Hartl *et al.*, 1994). In contrast to GroEL, very little is known about the mechanism and physiological substrates of the Class II chaperonins found in eukaryotic cells (Lewis *et al.*, 1996; Willison and Horwich, 1996). The TRiC complex is also ring shaped, but consists of eight different but homologous subunits ranging between 50 and 60 kDa (Frydman *et al.*, 1992; Gao *et al.*, 1992; Lewis *et al.*, 1992). TRiC does not require a GroES homolog but has been shown to cooperate with a recently described hetero-oligomeric complex called GIM or prefoldin (Geissler *et al.*, 1998; Vainberg *et al.*, 1998).

Despite recent advances in understanding the mechanism and general properties of eukaryotic chaperones, the overall contribution of cytosolic chaperones to *de novo* protein folding *in vivo* has not been defined (Ellis, 1996; Johnson and Craig, 1997). It is possible that only a few essential proteins require molecular chaperones to fold correctly, whereas the majority of proteins may not bind to chaperones during their biogenesis. Alternatively, all newly translated polypeptides may be channeled through a universal chaperone pathway, regardless of the properties of their folding intermediates.

Another poorly understood aspect of protein folding *in vivo* is the degree of functional integration between the different components of the chaperone machinery. Two models have been proposed to describe chaperone-mediated *de novo* folding (Bukau *et al.*, 1996; Ellis and

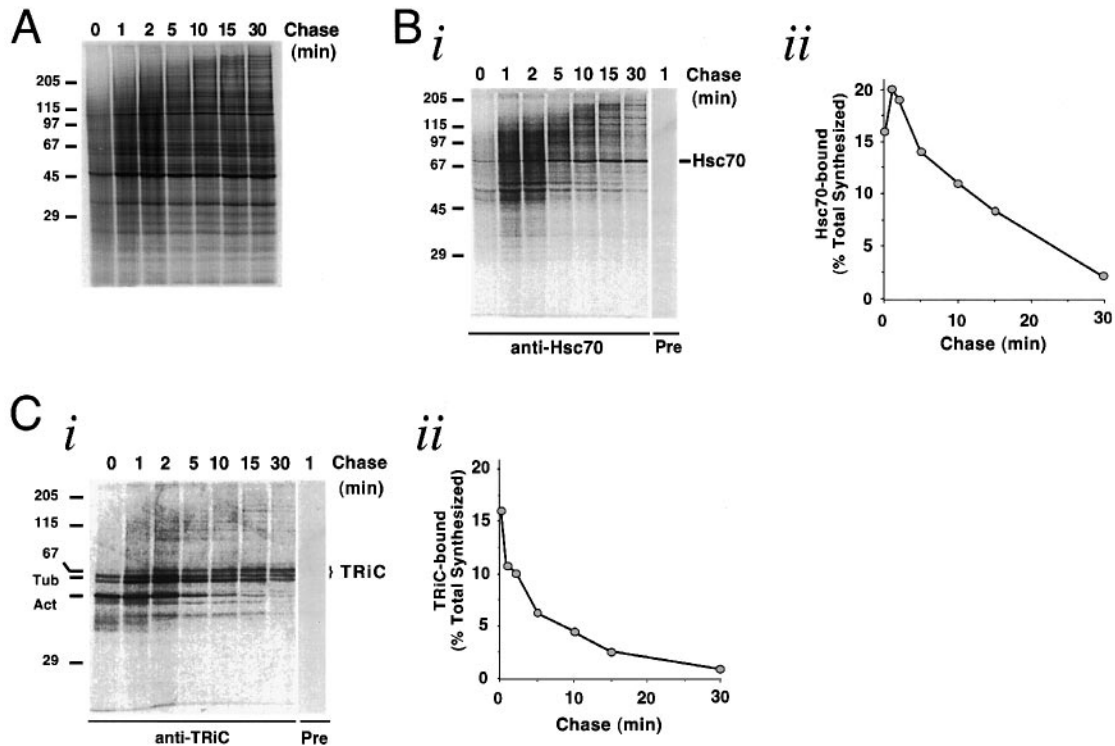


Fig. 1. Transit of newly synthesized proteins through cytosolic chaperones. BHK cells were pulse-labeled with [³⁵S]methionine for 60 s. Following a chase with non-radioactive methionine for the times indicated, the cells were lysed and the interaction with endogenous chaperones was assessed by immunoprecipitation. (A) Total proteins synthesized during the time course. (B) Time-course of polypeptide transit through Hsc70. SDS-PAGE (i) and quantitation (ii) of the Hsc70-associated polypeptides synthesized during the pulse-chase. (C) Time-course of polypeptide transit through TRiC. SDS-PAGE (i) and quantitation (ii) of the TRiC-associated polypeptides synthesized during the pulse-chase. Pre, pre-immune control.

Hartl, 1996; Frydman and Hartl, 1996; Farr *et al.*, 1997). According to the first, folding *in vivo* is assisted by a highly coupled chaperone machinery that interacts with polypeptides at an early stage while they are still ribosome-bound (Ellis and Hartl, 1996; Frydman and Hartl, 1996). In this model, the process of *de novo* folding is highly coordinated due to the processive interaction of the chaperone machinery with the intermediates of the folding reaction. This prevents the release of aggregation sensitive, non-native intermediates into the bulk cytosol. The other model proposes that, following translation, the unfolded polypeptides are fully discharged into the bulk cytosol (Bukau *et al.*, 1996; Farr *et al.*, 1997). The non-native polypeptides would then cycle between the bulk solution and the endogenous chaperones in a process that has been referred to as ‘kinetic partitioning’. In each cycle, a small fraction (~5%) of the polypeptides reaches the native state (Farr *et al.*, 1997) and the majority rebinds to chaperones. As a result, non-native folding intermediates partition between the solution and the chaperone systems present in the cytosol.

Here we have examined eukaryotic protein folding *in vivo*. Using intact mammalian cells we have directly analyzed the interaction of cytosolic chaperones with newly translated polypeptides. Pulse-chase analysis indicated that a large fraction of newly translated proteins associate transiently with Hsc70 and TRiC during their biogenesis. The substrate spectrum observed for Hsc70 and TRiC is not identical, consistent with the existence of multiple chaperone pathways which may reflect the distinct folding properties of different polypeptides. In

addition, we found that in mammalian cells molecular chaperones act in a highly processive manner. Our results are inconsistent with a stochastic model in which folding is achieved by the partitioning of non-native proteins between chaperones and the bulk cytosol. Rather, they provide support for the idea that newly synthesized polypeptides are prevented from drifting into the bulk cytosol throughout translation and folding, and reach the native state in a protected environment.

Results

Transit of newly translated proteins through cytosolic chaperones

The contribution of cytosolic chaperones to *de novo* folding was examined using pulse-chase analysis. Newly synthesized proteins were specifically labeled by subjecting cultured cells to a 60 s pulse with [³⁵S]methionine, followed by a chase with unlabeled amino acids (Figure 1A). Since we estimated the rate of elongation to be ~4 amino acids/s (see Materials and methods; Braakman *et al.*, 1991), this pulse is sufficient to synthesize a polypeptide of ~240 amino acids. Consequently, most of the labeled proteins can be completed and folded within the time course of the chase. At different time points during the chase, the cells were rapidly lysed under conditions that prevent ATP hydrolysis, in order to preserve the chaperone-substrate complexes. Our attention was focused on the interactions of nascent polypeptides with the cytosolic chaperone systems that have been implicated previously in *de novo* protein folding, namely Hsc70 and

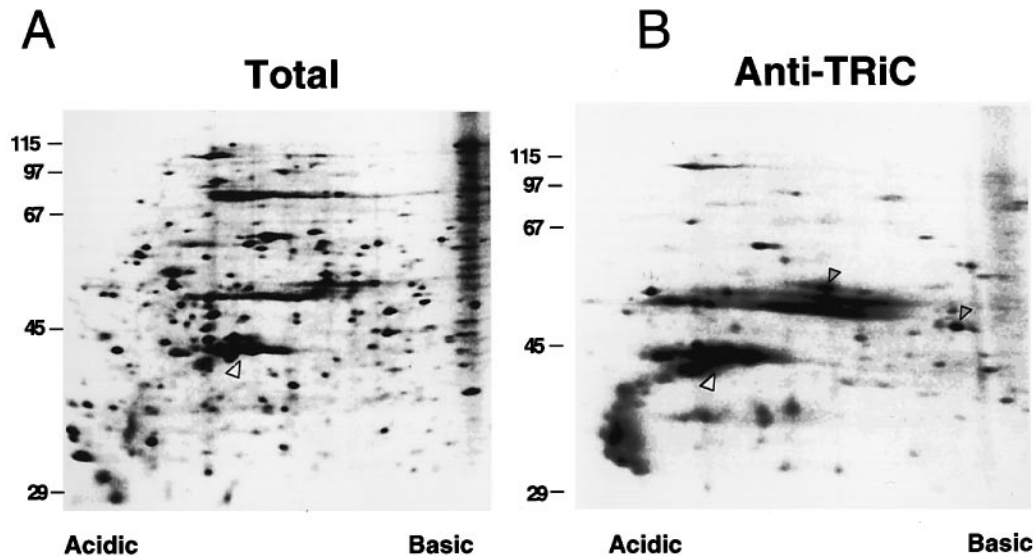


Fig. 2. Two-dimensional gel analysis of TRiC-bound newly synthesized proteins. Following a 90 s labeling pulse, the TRiC–substrate complexes were isolated by immunoprecipitation with antibodies specific for the β + ϵ subunits of TRiC and analyzed by two-dimensional gel electrophoresis. (A) Total proteins labeled during the pulse. The open arrow indicates the position of actin. (B) Newly synthesized proteins associated with TRiC. The gray small arrows indicate the positions of the newly synthesized, ^{35}S -labeled β - and ϵ -subunits of TRiC, which are not yet incorporated into the assembled complex.

the chaperonin TRiC. The fraction of chaperone-bound newly translated polypeptides was determined by co-immunoprecipitation with chaperone-specific antibodies (Figure 1B and C). As shown in Figure 1B, Hsc70 associated transiently with a broad spectrum of labeled polypeptides larger than 20 kDa. The maximal association was observed at early chase times (Figure 1Bii) and only a small fraction of labeled polypeptides, including newly synthesized [^{35}S]Hsc70, remained associated after a 30 min chase. As expected for Hsc70–substrate complexes, the presence of Mg/ATP during lysate preparation abrogated the interaction of Hsc70 with the labeled polypeptides (not shown). The interaction was established prior to cell lysis as inclusion of excess (20-fold) unfolded competitor polypeptide (reduced α -lactalbumin; see Palleros *et al.*, 1994) during lysis did not appreciably reduce the amount of labeled polypeptides associated with Hsc70 (not shown). The transient nature of the interaction and the sensitivity to the presence of Mg/ATP strongly suggest that these proteins are substrates of the chaperone. Quantitative analysis (see Materials and methods) indicated that ~15–22% of the polypeptides synthesized during the pulse were transiently bound to Hsc70 during their biogenesis (Figure 1Bii). A large fraction of these polypeptides were >50 kDa, suggesting that smaller proteins have either a more limited requirement or a weaker affinity for Hsc70.

The association of newly synthesized polypeptides with the chaperonin TRiC was examined in a similar manner (Figure 1C). Immunoprecipitations were performed using a mixture of antibodies specific for the C-termini of two subunits of TRiC (β + ϵ) which can recognize the assembled complexes (see 30 min chase; Hynes *et al.*, 1995). The spectrum of physiological substrates for this chaperonin has not been well defined: although it has been reported that the only substrates of the chaperonin are actin and tubulin (Eggers *et al.*, 1997), TRiC also appears to bind to firefly luciferase and $\text{G}\alpha$ -transducin (Frydman *et al.*, 1994; Farr *et al.*, 1997). As shown in Figure 1C,

several newly synthesized polypeptides, in addition to actin and tubulin, associated with TRiC and dissociated with different kinetics. Interestingly, the size of most polypeptides that transiently associated with TRiC ranged between 30 and 60 kDa, consistent with the idea that substrates of this chaperonin fold within a cavity which has an upper size limit of ~60 kDa (Ditzel *et al.*, 1998). However, several proteins of 100–120 kDa also associated transiently with TRiC, raising the interesting possibility that the chaperonin complex may be involved in the domain-wise folding of larger proteins.

The spectrum of polypeptides that interact with TRiC was further examined by two-dimensional gel electrophoresis (Figure 2). At least 70 different TRiC-bound polypeptides could be distinguished in this analysis (Figure 2B). The relative intensity of TRiC-associated proteins, particularly actin, reflects their relative abundance in the lysate (Figure 2A, open arrow points to actin). Importantly, the weaker signals produced by other TRiC-bound proteins may be due to their lower expression level since more abundant proteins tend to be favored in this analysis. Approximately 9–15% of the proteins labeled during the pulse were found to transit through TRiC (Figure 1C, right panel). It should be noted that the high stringency of the co-immunoprecipitation method may not allow detection of weakly bound or rapidly dissociated substrates, thus our quantitation only provides a lower estimate of the fraction of polypeptides that transit through chaperones *in vivo*.

Overexpression of the D87K-GroEL trap in mammalian cells

We next examined the degree of functional coupling between folding and translation. The main distinction between the two models proposed for chaperone-mediated folding lies in whether non-native intermediates are sequestered from the bulk cytosol during their biogenesis. The release of folding intermediates into solution can be

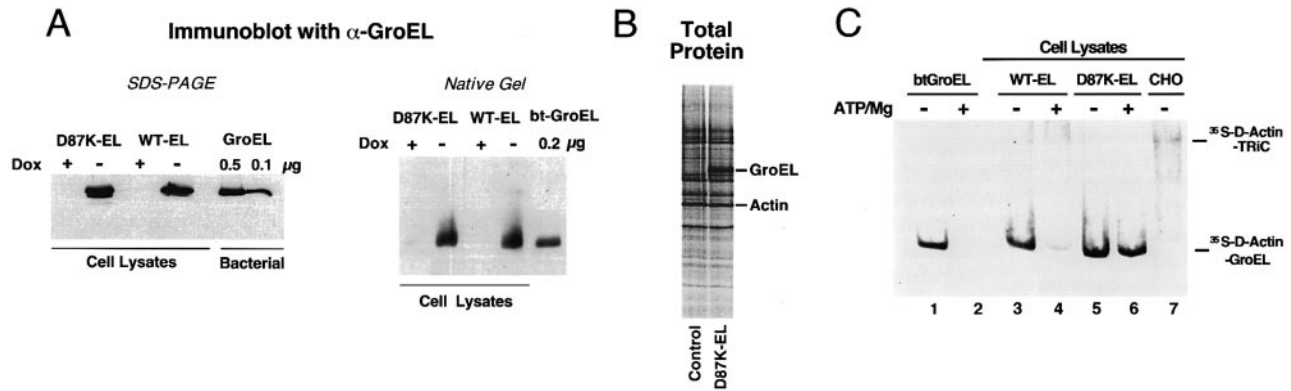


Fig. 3. Inducible overexpression of functional wild-type (WT) GroEL and D87K GroEL in mammalian cells. (A) Immunoblot analysis of cell extracts with anti-GroEL antibodies. GroEL expression was induced by removal of the repressor doxycycline (Dox). Induced and uninduced cell extracts (10 µg) were separated by SDS-PAGE (left panel) or native gels (right panel), and GroEL expression was monitored by immunoblot. The level of induction was quantified using pure bacterial GroEL (bt-GroEL) as a standard. (B) Steady-state protein synthesis in control and D87K-EL cells. Cells were labeled with [³⁵S]methionine for 20 min in the absence of Dox. The parental CHO cell line was used as the control. (C) The GroEL complexes expressed in mammalian cells are functional. [³⁵S]actin, denatured in 6 M guanidinium chloride (Gdm-HCl) was diluted 100-fold into lysates (50 µl, 0.5 mg/ml) from induced cells or control CHO cells. Equal aliquots of the sample were incubated in the presence or absence of ATP/Mg for 30 min at 30°C. Chaperone-[³⁵S]actin complexes were monitored by native gel electrophoresis and autoradiography. Binding of denatured [³⁵S]actin to purified bacterial GroEL (1 µg) was also tested as a positive control. The migration of GroEL and TRiC in the native gels were confirmed by immunoblot analysis.

assessed by introducing mutants of the bacterial chaperonin GroEL (such as D87K-GroEL) which act as ‘molecular traps’ since they bind irreversibly to non-native polypeptides (Weissman *et al.*, 1994; Frydman and Hartl, 1996; Farr *et al.*, 1997). Both models make distinct predictions on the effect of D87K-GroEL on *de novo* folding. If the chaperone machinery is highly organized and processive, the presence of trap should not affect the folding kinetics nor the interaction of newly synthesized polypeptides with endogenous chaperones. On the other hand, if newly synthesized polypeptides are discharged into the solution in a non-native state, the presence of trap should inhibit folding, as the non-native proteins would bind irreversibly to D87K-GroEL.

To discriminate between these two models we introduced the ‘molecular trap’ D87K-GroEL into intact mammalian cells. A tetracycline-inducible expression system (Gossen and Bujard, 1992; Freundlieb *et al.*, 1997) was used to generate stably transfected cell lines expressing either D87K-GroEL (D87K-EL) or wild-type GroEL (WT-EL). Upon induction, both WT-EL and D87K-EL were expressed at high levels (Figure 3A) and were correctly assembled, as judged by native gel electrophoresis (Figure 3A) and gel filtration chromatography of the cell lysates (not shown). Importantly, overexpression of D87K-EL trap was not toxic to cells as determined by cell growth and morphology (see below, Figure 4C). Quantitative analysis indicated that upon induction, both WT-EL and D87K-EL were expressed at similar levels and constituted ~3–6% (7.5–15 µM) of the total soluble proteins. D87K-EL was one of the major proteins expressed in the induced cells as indicated by SDS-PAGE analysis of total cellular proteins labeled in a 20 min pulse (Figure 3B). TRiC constitutes only ~0.2–0.4% (~0.5 µM) of the total soluble proteins (data not shown). Given the high affinity of D87K-EL for unfolded proteins (Farr *et al.*, 1997) the overexpressed D87K-EL trap should be an effective competitor of TRiC, as previously observed *in vitro* (Frydman and Hartl, 1996; Farr *et al.*, 1997; see also Figure 3C).

To determine whether the expressed GroEL complexes were functional in binding denatured proteins, extracts from GroEL-expressing cells were incubated with denatured [³⁵S]actin ([³⁵S]D-actin) in the presence or absence of Mg/ATP. The complex between cytosolic chaperones and [³⁵S]D-actin was visualized by native gel electrophoresis followed by autoradiography (Figure 3C). As expected, [³⁵S]D-actin binds to pure bacterial GroEL (Figure 3C, lane 1) and is released in the presence of Mg/ATP (lane 2). Both WT-EL and the trap D87K-EL expressed in the cells were fully capable of binding [³⁵S]D-actin (lanes 3 and 5). Actin was released from WT-EL by incubation with Mg/ATP (lane 4), whereas the [³⁵S]actin-D87K-EL complex remained unaffected under the same conditions (lane 6). Importantly, quantitative analysis indicated that between 60 and 90% of WT- and D87K-GroEL in the lysate were unoccupied and thus competent to bind the added substrate (not shown). When [³⁵S]D-actin was diluted into a lysate prepared from parental Chinese hamster ovary (CHO) cells (which do not express GroEL), only a very faint complex with endogenous TRiC was observed (Figure 3C, lane 7), highlighting the large excess of functional D87K-EL over endogenous chaperones in the cell extracts (compare lane 7 with lanes 3 and 5).

Expression of D87K-GroEL *in vivo* does not inhibit *de novo* folding

The effect of D87K-EL trap overexpression on the interaction of newly synthesized polypeptides with endogenous chaperones was now determined by pulse-chase analysis, as described for Figure 1 (Figure 4A and B). When compared with control cells, the presence of the trap had no effect on the association of newly translated proteins with either TRiC (Figure 4A) or Hsc70 (not shown). Moreover, the D87K-EL trap did not detectably bind any labeled polypeptides (Figure 4A), despite the higher levels of D87K-EL trap over endogenous chaperones. These results indicate that the folding intermediates of newly

synthesized proteins are not accessible to binding by the trap.

We next examined the effect of D87K-EL expression on the folding of actin, a model substrate for TRiC-mediated folding and one of the most abundant proteins in the cytosol (Figures 1A and 2). The kinetic partitioning model would predict a profound effect of D87K-EL on the folding of actin, and previous reports have indicated that microinjection of D87K-EL into *Xenopus* oocytes inhibits the production of folded actin (Farr *et al.*, 1997). Consequently, we examined the effect of D87K-EL on the rate and yield of actin folding *in vivo* (Figure 4B). Our kinetic analysis indicated that newly synthesized actin was already bound to TRiC at the earliest chase times and was released with a half time of ~50 s (Figure 4B). This release occurred concurrently with the appearance of folded actin, as determined by its ability to bind to DNase I-Sepharose beads (Zechel, 1980; Frydman and Hartl, 1996; Farr *et al.*, 1997). Importantly, neither the yield nor the kinetics of actin folding were altered in cells expressing D87K-EL as compared with control cells (Figure 4B). Previous measurements of the rate of actin transfer from TRiC to D87K-EL *in vitro* indicated that the half time of a single round of actin release from TRiC in a crude mammalian lysate is ~30 s (Farr *et al.*, 1997). During this 30 s time-frame only 5% of the released actin acquired the native state (Farr *et al.*, 1997). Thus, the rapid actin folding kinetics observed *in vivo* ($t_{1/2} = 50$ s) is not consistent with the model in which actin folding occurs through numerous rounds of release and rebinding.

The biochemical analysis indicated that the trap is unable to compete with TRiC in *de novo* folding. To ensure that the expressed WT-EL and D87K-EL were present in the cytosol, we examined their subcellular distribution using immunofluorescence (Harlow and Lane, 1988; Roobol *et al.*, 1995). Double immunofluorescence staining indicated that GroEL and TRiC colocalized in the cytosol (Figure 4C, upper panel). The integrity of the actin and tubulin cytoskeleton was also examined using rhodamine-phalloidin (to detect actin filaments) and anti-tubulin antibodies. There were no obvious abnormalities in the gross cytoskeletal morphology of cells expressing either GroEL variant (Figure 4C). We conclude that expression of WT-EL and D87K-EL does not adversely affect the folding environment in these cells.

D87K-GroEL can bind non-native proteins *in vivo*

The functional ability of D87K-EL trap to bind non-native polypeptides *in vivo* was tested in a series of control experiments. We reasoned that if a newly synthesized protein were unable to fold after being discharged from the coordinated chaperone pathway established during translation, it should be accessible to D87K-EL trap. Similarly, non-native polypeptides that were generated by a protein denaturing stress should also be available to D87K-EL trap because they too would be outside the chaperone/translation pathway. Initially, control and D87K-EL expressing cells were treated for 20 min with [³⁵S]methionine in the presence of 2-azetidinecarboxylic acid (AZC), an amino acid analog which is incorporated in place of proline. Proteins synthesized under these conditions are unable to fold and instead associate with Hsp70, as shown by co-immunoprecipitation (Figure 5A;

Beckmann *et al.*, 1990). When cells expressing D87K-EL were subjected to an identical treatment, the AZC-containing polypeptides readily associated with the D87K-EL trap (Figure 5A). Moreover, both the intensity and the pattern of polypeptides observed bound to the trap in the D87K-EL cells were comparable to that bound to Hsc70 in the parental AZC-treated cells (Figure 5A). Importantly, actin synthesized under these conditions was unable to fold (data not shown) and was now bound by D87K-EL, in contrast to actin synthesized under normal conditions (Figure 4A). This experiment demonstrates that the D87K-trap has the capacity to bind to non-native polypeptides, including actin, if they are released into the cytosol. Similar results were obtained when denatured polypeptides were generated by either heat shock (Figure 5B) or 8% ethanol treatment (Figure 5B) (Li and Werb, 1982). These conditions also resulted in actin denaturation, which was bound by the trap. We conclude that although the trap has no access to the intermediates generated during the productive folding of newly synthesized polypeptides, it is capable of binding non-native proteins generated *in vivo* if they are outside the protected folding environment established during translation.

Discussion

Substrate repertoire of cytosolic chaperones

We have examined the folding of newly synthesized proteins in intact mammalian cells and assessed the contribution of cytosolic chaperones to this process. During their biogenesis, a large fraction of newly translated proteins associate transiently with Hsc70 and TRiC. The substrate spectrum observed for Hsc70 and TRiC is not identical: Hsc70 interacts with a wide spectrum of polypeptides larger than 20 kDa while the substrate repertoire of TRiC comprises a more restricted subset of polypeptides ranging from ~30 to 60 kDa (Figures 1 and 2). It had previously been suggested that TRiC is a specialized chaperone that folds only a few cytoskeletal proteins (Willison and Horwich, 1996; Eggers *et al.*, 1997). This conclusion was based primarily on the analysis of TRiC mutants in *Saccharomyces cerevisiae* that are defective in actin and tubulin function (Chen *et al.*, 1994; Vinh and Drubin, 1994). However, these mutant phenotypes probably reflect the greater sensitivity of cells to alterations in the level of correctly folded actin and tubulin, which may obscure the effect of impaired folding of other TRiC-substrates. Also, previous studies using immunoprecipitation with TRiC-specific antibodies from metabolically labeled lysates resorted to very long labeling times (15 min in Eggers *et al.*, 1997) and lacked an ATP-depleting step to stabilize chaperone-substrate complexes. Given the rapid dissociation of most substrates from TRiC (see Figure 1), these conditions amount to a steady-state analysis, where only the most abundant (and tightly bound) substrates will be observed, in addition to the labeled chaperonin complex. By resorting to a careful kinetic analysis we find many additional polypeptides transiently associated with TRiC during their biogenesis (Figures 1 and 2). The 30–60 kDa size range observed for these TRiC-substrates lends further support to the idea that chaperonin-mediated folding occurs within an enclosed central cavity (Ditzel *et al.*, 1998). The identity and

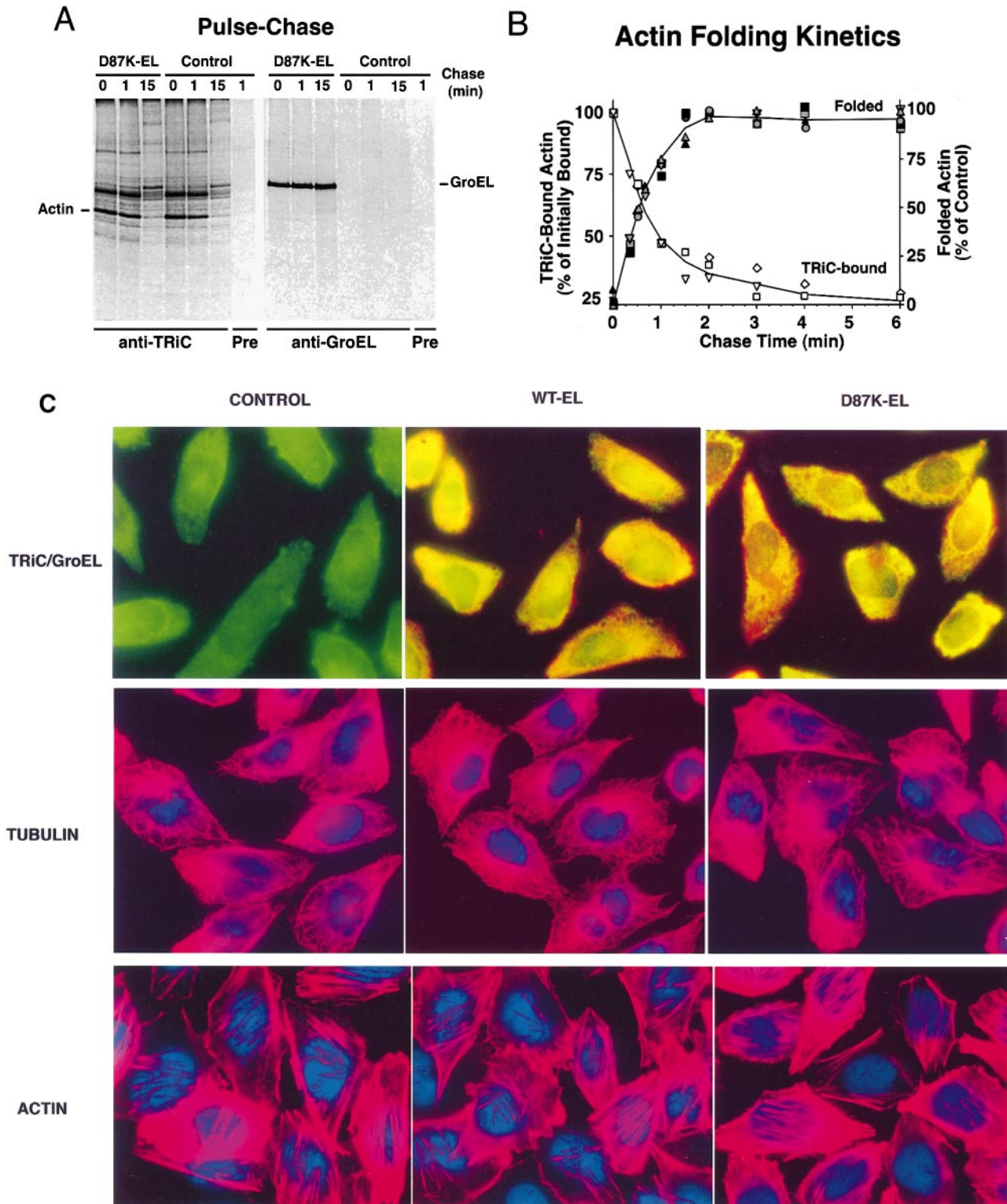


Fig. 4. *De novo* folding is not affected by the presence of high levels of D87K-GroEL in the cytosol. (A) The interaction of newly synthesized polypeptides with cytosolic chaperones is not affected by D87K-GroEL overexpression. Pulse-chase analysis of the interaction with TRiC in both control and D87K-EL expressing cells performed as in Figure 1. Binding of newly translated polypeptides to D87K-GroEL was also examined by immunoprecipitation. (B) High levels of GroEL-D87K do not affect the yield and kinetics of actin folding *in vivo* nor its dissociation from TRiC. Cells were pulse-labeled for 30 s and chased in the presence of 1 mM cycloheximide (see Materials and methods). Actin folding was measured in control cells (gray symbols) and in D87K-EL cells (black symbols). Dissociation from TRiC was measured in control cells (open symbols). Individual results from at least three separate experiments are shown. (C) Cells overexpressing WT-GroEL or D87K-GroEL have normal morphology. The distribution of GroEL and TRiC (upper panel) and tubulin (middle panel) were monitored by immunofluorescence with specific antibodies. The integrity of the actin cytoskeleton (lower panel) was monitored by staining with rhodamine-phalloidin. The nucleus was visualized by DAPI staining (blue). Cells in the upper panel were doubly stained to show the subcellular distribution of TRiC (green) and GroEL (red). Areas of overlap are yellow. Note that GroEL, unlike TRiC, does not enter the nucleus.

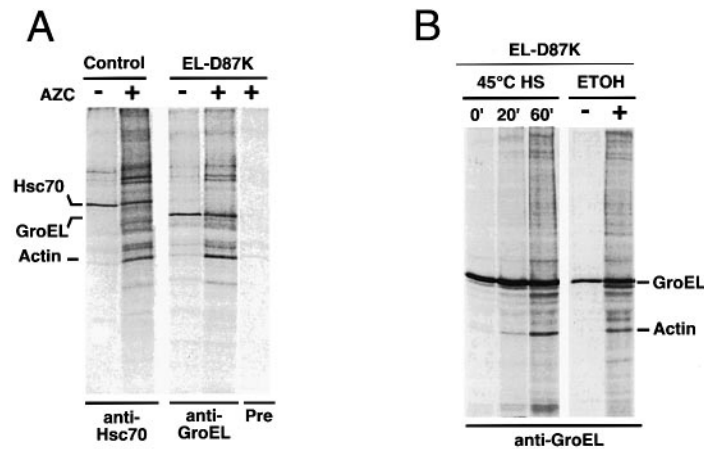


Fig. 5. D87K GroEL trap can bind non-native polypeptides generated *in vivo*. (A) D87K-EL binds non-native polypeptides that incorporated the proline analog AZC. Misfolded proteins were generated by treatment of D87K-EL cells with AZC for 20 min in the presence of [³⁵S]methionine; the association with the D87K trap was then examined by immunoprecipitation. The longer labeling time used in this experiment (20 min compared with 1 min in Figure 4A) resulted in a slightly higher level of background signal. Identical results were obtained following a 3 min pulse. Control cells were subjected to a similar treatment and the previously reported association of misfolded proteins with Hsp70 was confirmed by immunoprecipitation (Beckmann *et al.*, 1990). (B) GroEL trap binds to stress-denatured proteins. D87K-EL cells labeled for 20 min with [³⁵S]methionine were subjected to stress treatments in the presence of excess unlabeled amino acids. Protein denaturation was induced by incubation at 45°C for the times indicated or by incubation for 1 h at 37°C in media containing 8% ethanol. As a control, cells were incubated for 1 h at 37°C.

structural features that characterize these TRiC substrates have not been defined but, based on the structure of known examples, they may have a complex domain organization that results in folding intermediates with a higher tendency to aggregate (Kabsch *et al.*, 1985; Conti *et al.*, 1995; Sonddek *et al.*, 1996; Nogales *et al.*, 1998). We observed that ~15–20% of the total labeled proteins bound transiently to Hsc70 and ~9–15% associated with TRiC. Given the high stringency of the immunoprecipitation method, this is probably an underestimate of the fraction of cellular proteins that fold in a chaperone-assisted manner.

Folding of newly translated polypeptides in a sequestered environment

The processivity of the folding machinery *in vivo* was examined by overexpression of a GroEL-derived trap. GroEL binds promiscuously to the solvent-exposed hydrophobic regions present in non-native proteins and absent in correctly folded proteins (reviewed in Hartl, 1996; Bukau and Horwich, 1998). When expressed in the cytosol of mammalian cells, D87K-EL trap has no access to the folding intermediates generated during protein synthesis. These associate instead with the endogenous cytoplasmic chaperones (Figure 4), as would be expected if the chaperone machinery is tightly coupled to translation. It is striking that we do not observe any subset of newly translated polypeptides binding to D87K-EL. The fact that all newly synthesized polypeptides, including those that do not interact with Hsc70 or TRiC, are inaccessible to D87K-EL indicates that most folding intermediates generated during *de novo* folding in mammalian cells are unable to drift into the bulk cytosol. Importantly, this may reflect a general property of the cellular folding machinery whereby nascent polypeptides emerging from the ribosome either immediately adopt a conformation that prevents the exposure of hydrophobic regions or associate with molecular chaperones.

In contrast to proteins emerging from the ribosome, D87K-EL expressed in cells was fully capable of binding

stress-denatured and misfolded proteins (Figure 5). This result suggests that these proteins are not sequestered by the chaperone pathway established during *de novo* folding and can thus be bound by the D87K-EL trap. It has been recently reported that the chaperone-mediated reactivation of stress-denatured proteins also occurs in a partially sequestered environment (Glover and Lindquist, 1998). Thus, D87K-EL may compete with the endogenous ‘rescue systems’ only for the initial binding to denatured polypeptides. Interestingly, cells overexpressing WT-EL and D87K-EL were protected from heat-induced cell death (C.-F. Yang and J. Frydman, manuscript in preparation), suggesting that these high levels of GroEL variants can prevent the formation of toxic protein aggregates.

Our experiments also provide insight into the degree of sequestration of newly translated proteins that are incapable of reaching the native state. Cellular proteins synthesized in the presence of the proline analog AZC were unable to fold correctly and were now accessible to the D87K-EL trap. Thus, folding-incompetent polypeptides are released from the co-translationally established folding pathway and presumably, partition between endogenous chaperones and the ubiquitination machinery (Frydman and Hartl, 1996). Indeed, in control cells that do not express D87K-EL, the AZC-containing proteins bind to Hsc70 (Figure 5A) and are subsequently degraded (C.-F. Yang and J. Frydman, unpublished data).

Analysis of the effect of D87K-EL on the folding kinetics of actin further confirmed that *in vivo*, chaperone-mediated folding occurs in a sequestered environment. First, high concentrations of D87K-EL in the cytosol did not affect the production of folded actin. Moreover, the appearance of native actin *in vivo* was dramatically faster than predicted by a model involving inefficient cycles of binding and release. Thus, assuming that the kinetic parameters of substrate release measured in reticulocyte lysate reflect those *in vivo*, this model proposes that only 5% of the chaperonin-bound actin folds in each round of ATP-driven release, that is every 30 s (Farr *et al.*, 1997).

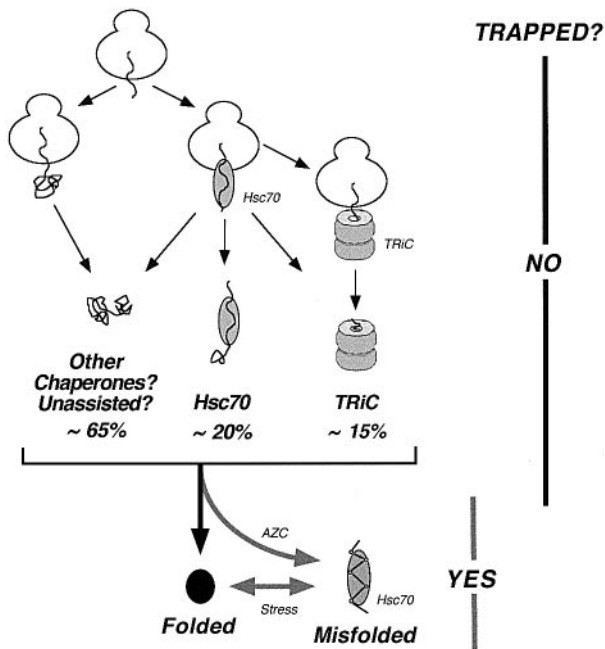


Fig. 6. Schematic model for *de novo* protein folding in the cytosol of mammalian cells. Nascent polypeptides emerging from the ribosome either immediately adopt a conformation that prevents the exposure of hydrophobic regions or associate with molecular chaperones. The initial interaction is probably with Hsc70, which may be recruited through interactions with the ribosome or other chaperone cofactors. The folding properties of individual proteins may determine whether this interaction persists upon completion of translation or whether the polypeptide will associate co- or post-translationally with the chaperonin TRiC. Specific subsets of polypeptides may be transferred to other chaperone systems, such as Hsp90 (not shown here), or may fold in a chaperone-independent manner. Chaperone interactions that are coupled to translation and lead to productive folding are shown in solid black arrows. The processive nature of this pathway renders newly made polypeptides inaccessible to the heterologous trap D87K-EL. Upon misfolding or stress-induced denaturation, non-native polypeptides may partition between chaperone-bound and free states and are thus bound by the trap. These interactions are shown with gray arrows.

Thus, the predicted half-time of actin folding according to this model would be ~6.7 min, while the half-time observed *in vivo* was 50 s.

Multiple chaperone pathways in *de novo* folding

Based on our results, we propose a model for chaperone-assisted and unassisted folding in the eukaryotic cytosol (Figure 6). In this model, ~20% of newly translated proteins transit through Hsc70 and ~15% transit through TRiC. Our results imply that these chaperones are specifically recruited to bind newly translated proteins, to the exclusion of the overexpressed bacterial chaperone (Figure 6, black arrows). Mechanistically, this could be achieved by the co-translational interaction of the chaperone machinery with nascent chains. In support of this notion, several studies have indicated that a number of components of the folding machinery are associated with translating ribosomes or nascent chains (Beckmann *et al.*, 1990; Zhong and Arndt, 1993; Frydman *et al.*, 1994; Wiedmann *et al.*, 1994; Pfund *et al.*, 1998). These include Hsc70 and Hsp40 homologs as well as the nascent chain associated complex (NAC), although the role of the latter in folding is unclear. Hsc70 may be the ‘gateway’

to the cytosolic chaperone machinery, being the first general chaperone system to contact nascent chains in the context of the ribosome. Interestingly, a recent study demonstrated that in yeast, the Hsc70 homolog SSB interacts with both the ribosome and the nascent chains (Pfund *et al.*, 1998). This interaction may be determined by the action of zuotin: an Hsp40 homolog that binds directly to ribosomes (Yan *et al.*, 1998) and may thus couple translation to the chaperone machinery. The ability of Hsc70 to interact with many cofactors, including various Hsp40 homologs, TPR-containing proteins such as Hip, Hop and Bag1 may play an important role in directing a bound substrate along a specific chaperone pathway (Cyr *et al.*, 1994; Ziegelhoffer *et al.*, 1996; Frydman and Hohfeld, 1997; Hohfeld, 1998). *In vitro* studies indicate that TRiC also binds co-translationally to substrates (Frydman *et al.*, 1994; Dobrzynski *et al.*, 1996; Frydman and Hartl, 1996) via an initial interaction with Hsc70 (Figure 6). However, it remains to be established whether the initial interaction with Hsc70 is a general requirement for all substrates of TRiC.

The fact that newly translated polypeptides remain in a sequestered environment until they have reached a conformation that no longer possesses extensive solvent-exposed hydrophobic regions and is close to the native state implies that the interaction of newly translated proteins with chaperones is highly processive. It will be interesting to understand how this processivity is achieved. In principle, ancillary cofactors, such as the GIM-prefoldin complex (Geissler *et al.*, 1998; Vainberg *et al.*, 1998), may play a role in establishing the tight coupling observed for both Hsc70- and TRiC-mediated folding.

What determines the chaperone interactions of a given polypeptide? Following an initial co-translational interaction with Hsc70, the folding properties of individual proteins may determine whether this interaction persists upon completion of translation (Figure 6). The broad size range of polypeptides associated with Hsc70 (Figure 1) suggests that the ability of this chaperone to prevent aggregation suffices to fold a large subset of polypeptides. Interestingly, most of the polypeptides that transit through Hsc70 are >50 kDa. Since the size of individual domains in cytosolic proteins is ~25–30 kDa, the substrates of Hsc70 probably include multi-domain proteins that are folded co-translationally (Figure 6). Our data also indicate that a considerable number of polypeptides require the protected folding environment provided by the chaperonin TRiC (Figures 1 and 2). These proteins may have a more complex, aggregation-prone domain structure, which may require an obligate post-translational folding step. It is noteworthy that the *Escherichia coli* chaperonin, GroEL, has a much broader substrate spectrum in the bacterial cytosol than observed for TRiC in eukaryotes (Figure 2; Ewalt *et al.*, 1997). This would be consistent with the proposal that most prokaryotic proteins fold in a post-translational manner (Netzer and Hartl, 1997). Thus, there may be important differences between prokaryotic and eukaryotic folding, both in terms of the interactions of the folding machinery with the translational apparatus and in the processivity of the chaperone machinery itself.

Our data also suggest that a significant portion of newly synthesized polypeptides (~65%) appear to reach the native state without the assistance of Hsc70 or TRiC

(Figure 6). It is conceivable that a fraction of these polypeptides folds in a chaperone-independent manner. This may be particularly true for small proteins and for large multi-domain proteins composed of small independent modules that can fold co-translationally (Netzer and Hartl, 1997). In addition, otherwise uncharacterized chaperone systems may be responsible for folding specific subsets of cytosolic proteins. For example, Hsp90 does not appear to play a general role in *de novo* folding (Nathan *et al.*, 1997), but is required for folding a restricted class of proteins that includes steroid hormone receptors and Src-like tyrosine kinases. Interestingly, these substrates have also been reported to require an initial interaction with Hsc70 prior to transfer to Hsp90 (reviewed in Frydman and Hohfeld, 1997). It is tempting to speculate that the translational machinery could also possess some chaperone-like functions such as prevention of aggregation (Kudlicki *et al.*, 1997; Caldas *et al.*, 1998), although this idea awaits further investigation.

Folding in the cell has to be achieved in a highly crowded macromolecular environment, whereby the release of non-native polypeptides into the solution might lead to the formation of potentially toxic aggregates. Consequently, the coordinated action of the chaperone machinery during translation may be required to protect folding intermediates from non-productive interactions (Ellis, 1997). The specific recruitment of molecular chaperones to bind newly translated polypeptides may also prevent the inappropriate targeting of incomplete nascent chains to the degradative apparatus. Importantly, our experiments using AZC incorporation indicate that there are mechanisms that allow the exit of mutated or damaged polypeptides from the translation/folding pathway. It is not clear how these polypeptides are released from the chaperone machinery to be degraded. In principle, this could be determined by kinetic partitioning of non-native proteins between chaperones and components of the ubiquitination machinery. Alternatively, some components of the folding machinery may play a role in directly targeting proteins to degradation. Elucidation of the molecular principles underlying this process will prove critical for understanding how the fate of a polypeptide is determined in the cell.

Materials and methods

Cell culture

Baby hamster kidney (BHK-21) cells were cultured in Glasgow medium supplemented with 20 mM HEPES (pH 7.2), 2.95 g/l tryptose phosphate, 5% FCS. CHO AA8-tet-off cell line (Clontech) were grown in the alpha-minimum essential medium (Gibco-BRL) containing 5% tetracycline-free FCS (Clontech). To generate stable single-cell clones expressing GroEL (wild-type or the D87K mutant), the open reading frames were cloned into pTRE (Clontech), introduced into AA8-tet-off cells (Clontech) using lipofectamine-Plus (Gibco-BRL) and selected as advised by the manufacturer (Clontech) (Gossen and Bujard, 1992; Freundlieb *et al.*, 1997), using 400 µg/ml hygromycin, 100 µg/ml G418 in addition to 2.5 ng/ml doxycycline (Dox) to inhibit GroEL expression. Single-cell cloning was performed by serial dilution and plating. GroEL expression was induced by removal of Dox for 48 h prior to analysis (Gossen and Bujard, 1992; Freundlieb *et al.*, 1997). Stably transfected HeLa-tet-off cells expressing both GroEL variants were also generated, and behaved exactly as shown here for CHO cells.

To generate misfolded proteins *in vivo*, cells were preincubated with the proline analog AZC (10 mM) for 30 min to increase the intracellular AZC to proline ratio and then labeled in the presence of 10 mM AZC

with [³⁵S]methionine (0.2 mCi/ml) for 20 min followed by a 30 min chase with unlabeled amino acids (Beckmann *et al.*, 1990). Alternatively, cells labeled for 20 min with [³⁵S]methionine were subjected to either heat shock at 45°C or incubation with 8% ethanol (Li and Werb, 1982).

Pulse-chase analysis was performed at 37°C. Cells (~1.0×10⁶) were incubated in methionine-free MEM media for 15 min, labeled with MEM media supplemented with [³⁵S]methionine and cysteine (0.4 mCi/ml) and chased with DMEM supplemented with 50 mM methionine and cysteine in the absence (Figures 1 and 4A) or presence (Figure 4B) of 1 mM cycloheximide. The incorporation of [³⁵S]methionine stopped within 10 s of adding the unlabeled amino acids, and all subsequent time-points had the same specific activity when measured as TCA-insoluble c.p.m./mg protein. The apparent increase in label observed in Figure 1A between 0 and 1 min is due to the migration of short, labeled polypeptides with the gel front. At the indicated times, cells were placed on ice and incubated with ice-cold ATP depleting solution (PBS containing 1 mM NaN₃, 2 mM 2-deoxyglucose (2-DOG), 5 mM EDTA and 5 mM cycloheximide). Cells were scraped and lysed as described below.

For immunofluorescence analysis, cells were grown on 12 mm coverslips, fixed with freshly prepared 4% paraformaldehyde in 20 mM HEPES-KOH (pH 7.4) for 15 min at 25°C and permeabilized with either 0.1% Triton X-100 in PBS or methanol at -20°C, for 5 min. Staining was performed exactly as described (Harlow and Lane, 1988; Roobol *et al.*, 1995). GroEL variants were detected using a monoclonal antibody (mAb) with no cross-reactivity with mammalian Hsp60 (SPA-870, Stressgen). TRiC was detected using rabbit affinity-purified subunit-specific antibodies raised against the C-terminus of the β- and ε-subunits (also used for immunoprecipitation, a generous gift of Martin Carden, Kent University, UK) (Hynes *et al.*, 1995; Roobol *et al.*, 1995) and tubulin with a mixture of mAbs to the α+β subunits (Sigma). Actin filaments were visualized with rhodamine-phalloidin (Sigma). Nuclei were visualized by staining with 4',6'-diamidino-2-phenylindole (DAPI). Secondary antibodies were affinity-pure goat anti-mouse-Texas Red and goat anti-rabbit-FITC conjugates (Sigma).

Biochemical techniques

Cell lysates were prepared by dounce homogenization (100 strokes) in buffer A (25 mM HEPES-KOH pH 7.4, 100 mM KCl, 5mM EDTA, 1 mM DTT, 0.1 mM PMSF) and clarified by high-speed centrifugation (20 min at 20 000 g). For pulse-chase experiments, the buffer also contained 1 mM DOG, 1 mM NaN₃ and 50 µg/ml digitonin. Binding of [³⁵S]D-actin to the expressed GroEL variants, native gel electrophoresis, Western blot analysis and Superose 6 gel filtration chromatography were performed exactly as described (Frydman *et al.*, 1994; Frydman and Hartl, 1996). The extent of actin folding in the lysates was determined by binding to DNase I-Sepharose beads as described (Zechel, 1980; Frydman and Hartl, 1996; Farr *et al.*, 1997).

For the two-dimensional gel analysis, samples in isoelectrofocusing (IEF) buffer (3.2M urea, 0.7% Nonidet P40, 0.5% ampholines pH 5-7, 0.16% ampholines pH 3-10, 1.6% 2-mercaptoethanol) were loaded on IEF tube gels (1% ampholines pH 3-10 and 4% ampholines pH 5-7) and separated exactly as described (Jones, 1984). Ten percent SDS-PAGE gels were used for the second dimension. The position of actin was confirmed by Western blot analysis.

Immunoprecipitation and quantification

The elongation rate was calculated based on the rate of Hsc70 and actin synthesis as described in Braakman *et al.* (1991). Cell lysates (20 µl, normalized to 0.1 mg/ml) were incubated for 45 min on ice, with the addition of bovine serum albumin (BSA) (10 mg/ml) and antibodies against TRiC (3 µl, rabbit affinity purified), Hsp70 (4 µl, rabbit polyclonal) (Frydman *et al.*, 1994), GroEL (5 µl, mAb SPA-870, Stressgen) or pre-immune (3 µl, rabbit). Immune complexes were collected by further incubation with protein A (or protein G)-Sepharose beads (10 µl in BSA, 10 mg/ml) for 45 min at 4°C with gentle motion. After incubation the beads were washed twice with both TBS-0.05% Tween 20 and TBS-1% Tween 20.

The fraction of the total ³⁵S-labeled proteins associated with each chaperone at a given time point was calculated as the ratio of total [³⁵S]protein co-immunoprecipitated with that chaperone (quantified by PhosphorImager analysis, Molecular Dynamics) divided by the total amount of [³⁵S]protein in the lysate of the corresponding time-point (quantified by PhosphorImager analysis of an SDS-PAGE of the total). Our estimation incorporated the efficiency of immunoprecipitation (~65% for TRiC and ~90% for Hsc70), which was measured by performing a second immunoprecipitation on the supernatants for the immune and

non-immune reactions. The stability of substrate-chaperone complexes to the immunoprecipitation and wash procedure was also measured by subjecting pre-isolated chaperone-substrate complexes to the immunoprecipitation procedure (~20% loss of Hsc70 substrates and ~35% loss of TRiC substrates). We found that TRiC-substrate complexes were very sensitive to the time elapsed between cell-lysis and completion of all the procedures, even at 4°C and in the absence of ATP. The levels of [³⁵S]actin in the TRiC immunoprecipitations, bound to DNase I-Sepharose beads and in the total, were quantified directly from SDS-PAGE gels. This reflects accurately the total amount of actin in the sample since 2D-gel analysis indicated that actin constitutes >98% of the ³⁵S label in that molecular weight range.

Acknowledgements

We thank Mark Mayhew, Raul Andino, Damian McColl and William Netzer for discussions and for comments on the manuscript, and Carol Gross for suggesting ethanol treatment as a denaturing stress. We also thank Martin Carden for his generous gift of anti-TRiC antibodies, Ron Kopito and Martha Cyert for comments on the manuscript, Dan Howard for help in single-cell cloning, Jen Johnson for advice on immunofluorescence and Pat Jones for advice on two-dimensional gels. J.F. is supported by NIH grant GM56433.

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Received October 6, 1998; revised and accepted November 25, 1998